Virus-induced gene silencing in Streptocarpus rexii (Gesneriaceae)

Title

Virus-induced gene silencing in *Streptocarpus rexii* (Gesneriaceae)

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Abstract

Many members of the family Gesneriaceae are cultivated as ornamental plants, including Cape primrose (*Streptocarpus*) species. The range of plant architecture found in this genus has also made it a model to study leaf and meristem development and their evolution. However, the lack of tools to study gene functions through reverse genetics in *Streptocarpus* has limited the exploitation of its genetic potential. To aid functional genomic studies in *Streptocarpus rexii*, we sought to investigate virus-induced gene silencing (VIGS). Using the broad host range Tobacco Rattle Virus (TRV) to target the *PHYTOENE DESATURASE* (*PDS*) gene of *S. rexii*, we show that infection with sap from *Nicotiana benthamiana* triggered VIGS efficiently. VIGS was most effective in the seedling leaves 8 weeks after sowing, but was limited in duration and systemic spread. This study reports the first successful use of VIGS in *Streptocarpus* and in the family Gesneriaceae. The inoculation of viral sap derived from *N. benthamiana* was able to overcome the difficulties of standard Agrobacterium-mediated transformation in this genus. Irrespective of its transient effect, this VIGS system will be useful to assess gene function at the cellular level and represent an important tool for further understanding molecular mechanisms in *Streptocarpus*.

Keywords Gesneriaceae· *Nicotiana benthamiana*· *PHYTOENE DESATURASE*· *Streptocarpus rexii*· Tobacco rattle virus· Virus-induced gene silencing

Background

The Cape primrose *Streptocarpus rexii* (Gesneriaceae) lacks a conventional shoot system and shoot apical meristem. It produces leaves, termed phyllomorphs, and inflorescences from intercalary meristems at the base of preceding phyllomorphs. Each phyllomorph consists of a lamina and a stem-like petiole, the petiolode. Growth of the phyllomorph is basipetal; it retains a meristem in its proximal region that adds new cells to the base of the growing lamina. It also has a groove meristem at the juxtaposition of the lamina and petiolode, which forms new phyllomorphs and a rosulate (false rosette) morphology, and a petiolode meristem that allows thickening and elongation of the petiolode [1]. Another feature of *Streptocarpus* species, common to most Old World Gesneriaceae, is anisocotyly [1, 2], where growth of one cotyledon ceases and the other continues to expand via the basal meristem [3, 4]. In acaulescent *Streptocarpus* species, including the rosulate *S. rexii*, the larger cotyledon (also referred to as macrocotyledon) becomes the first phyllomorph, or the only phyllomorph in unifoliate *Streptocarpus* [1]. In contrast, the smaller cotyledon (microcotyledon) does not grow further and later withers away. *Streptocarpus rexii* has been established as a model to study the evolution of the leaf and shoot functions, and has available genomic resources, such as a transcriptome database and a genetic map (e.g. [5, 6]). However, the lack of reverse genetics tools limits the exploitation of its genetic potential.

Virus-induced gene silencing (VIGS) is a useful tool for studying the role of genes through loss-of-function, particularly in species that are recalcitrant to genetic transformation [7]. It involves triggering of the plant's antiviral immune system with an RNA sequence homologous to an endogenous transcript, resulting in RNA silencing of both the virus genome and the endogenous gene [8–10].

Tobacco rattle virus (TRV) has a broad host range and has proved useful as a VIGS vector in a range of eudicots and basal angiosperms [7], including ornamentals (e.g. [11]). Its positive-strand RNA genome becomes double-strand RNA (dsRNA) when replicated in the plant host, triggering the plant immune system, which cleaves dsRNA into small interfering RNA (siRNA). siRNAs are incorporated into the RNA-induced silencing complex (RISC), which targets RNAs complementary in sequence to siRNAs [12]. The TRV has a bipartite positive RNA genome consisting of RNA1 (TRV1) and RNA2 (TRV2) [13, 14]. TRV1 is essential for viral movement and replication, while TRV2 has genes encoding the viral coat protein and non-structural proteins. To utilise the TRV VIGS system for targeted gene silencing, a partial target gene sequence is inserted within a TRV2 cDNA in the pTRV2 vector, and delivered along with pTRV1 by Agrobacterium-mediated transformation of the host (often the highly susceptible *Nicotiana benthamiana*), resulting in expression of both viral RNAs and assembly of infective virus particles [14, 15]. Infectious viral sap from *N. benthamiana* can then be used to inoculate the target species by mechanical damage [16].

In this study, we investigated the application of TRV-mediated VIGS in *Streptocarpus rexii* in order to establish a much sought after reverse genetic tool in this species. We used the *Streptocarpus phytoene desaturase* (*PDS*) gene as the reporter gene. Its silencing in VIGS experiments results in a photobleaching phenotype [17]. We found that TRV can induce transient gene silencing in *S. rexii* phyllomorphs (for simplicity termed phyllomorph as leaf hereafter), which opens new avenues for functional genomic studies in Gesneriaceae.

Materials and Methods

Plant Material

Streptocarpus rexii (RBGE accession number ex-19870333) seedlings were used in this study. This species is genetically homogenous in the wild due to high level of inbreeding [18]. Plants of this accession have been in cultivation at RBGE for more than 30 years, and the short-lived plants are frequently propagated sexually through self-pollination, and are likely to be highly homozygous. The genome heterozygosity of the parental *S. rexii* plant, of which the progeny was in this study, was estimated as 0.05–0.06% [6]. Seeds were germinated in Levington F2 + S professional growth compost in 7 × 7 × 6 cm pots sealed in plastic bags to maintain high humidity, at 22 °C in 16 h light 8 h dark cycle. Seedlings were transplanted to individual pots 5–10 weeks after sowing (WAS) and maintained under the same conditions. *Nicotiana benthamiana* seeds were germinated in Levington F2 compost in a controlled growth chamber (Sanyo, Osaka, Japan) at 22 °C with 16 h light and 8 h dark periods. *S. rexii* plants were inoculated between 5 and 10 WAS.

Isolation of *Streptocarpus rexii PHYTOENE DESATURASE* Gene

A *PDS* homolog, *SrPDS* was identified by BLAST searching the *S. rexii* transcriptome database (https ://elixi r-italy.org/milan o/en/archi ves/servi zi/angel dust-1-0) [5]. Primers were designed to amplify a region of 416 bp close to the 3′ end of the open reading frame that was conserved between *N. benthamiana* and *S. rexii* [17]. Primer sequences are shown in Table 1 and their positions are indicated in Figs. 1a and S1.

pTRV2^{SrPDS} Vector Construction

To amplify the *SrPDS* target, RNA was extracted from leaves of 3 months old *S. rexii* plants using Trizol (Invitrogen, Carlsbad, CA, USA), subjected to extraction with phenol:chloroform (5:1, pH 4.3–4.7; Sigma, St. Louis, MO, USA) and precipitated with isopropanol before being further purified with a Purelink™ RNA Mini kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. cDNA was synthesized with SuperScript III reverse transcriptase (Thermo Fisher Scientific) and PCR was carried out with the *SrPDS* primers with Q5® High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA). Amplified DNA was purified with a MinElute Gel Extraction kit (Qiagen, Hilden, Germany) and phosphorylated with T4 polynucleotide kinase (Thermo Fisher Scientific). The pTRV2 vector was digested with *Sma* I and dephosphorylated with Shrimp Alkaline Phosphatase (New England Biolabs), before ligation with the *SrPDS* sequence using T4 DNA ligase. The plasmids were used to heat shock transform *Escherichia coli* (DH5α), selected with kanamycin (50 μg/ml) and colonies screened by PCR with *SrPDS* primers. Plasmids were Sanger sequenced from the TRV-CP backbone primer (Table 1) at Edinburgh Genomics (University of Edinburgh). Antisense *SrPDS* inserted pTRV2 vector (pTRV2^{SrPDS}) was used in further steps.

Agrobacterium Infiltration

The Agrobacterium strain GV3101-pMP90-pSOUP was transformed with the pTRV1, pTRV2^{WT}, or pTRV2^{SrPDS} vectors separately using an Electroporator 2510 (Eppendorf, Hamburg, Germany) at 1800 V and cuvettes with a 1 mm electrode gap. Transformed cells were precultured in 450 μl YEP medium at 28 °C and selected on YEP agar plates containing kanamycin 50 μg/ml, gentamycin 25 μg/ml, and rifampicin 50 μg/ml. Bacterial colonies were precultured in 5 ml liquid YEP medium containing kanamycin 50 μg/ml, gentamycin 25 μg/ml, and rifampicin 50 μg/ml overnight in 15 ml Falcon tubes and 0.2 ml of culture used to inoculate 10 ml of fresh YEP medium supplemented with the above antibiotics for overnight culture.

Cultures were centrifuged at 4000 rpm (3095 × *g*) for 20 min at room temperature. Supernatants were removed and pellets suspended in infiltration buffer (10 mM MES pH5.6, 150 μM acetosyringone, 10 mM $MgCl_2$) and adjusted to an OD600 of 1.0 with infiltration buffer. After incubation at room temperature for 2–3 h, the GV3101:pTRV1 suspension was mixed with an equal volume of either GV3101:pTRV2^{WT} or GV3101:pTRV2^{SrPDS} for infiltration of *S. rexii* or *N. benthamiana*.

Approximately 500 μl of bacterial suspension was infiltrated with a 1 ml needleless syringe into the abaxial leaf surface 4 weeks after sowing (WAS) for *N. benthamiana* or 5 to 10 WAS for *S. rexii*

[16]. For *N. benthamiana*, three leaves 5–10 cm in length were infiltrated per plant. For *S. rexii*, only the macrocotyledon was infiltrated, as it was only visible leaf at that stage. The length of macrocotyledons at 5, 8, and 10 WAS was 9.3 ± 0.3 mm, 18.7 ± 1.2 mm, and 33.2 ± 2.0 mm, respectively (average ± standard error, $N = 10$). After infiltration, plants were placed under a transparent propagator lid to maintain high humidity. Lids were removed two days after infiltration of *N. benthamiana*, but kept on throughout the experiment for *S. rexii*.

Harvesting *N. benthamiana* Plant Sap Containing TRV^{WT} and TRV^{SrPDS}

One week after infiltration, young *N. benthamiana* leaves showing viral symptoms were harvested and ground in 1 mM sodium phosphate buffer (pH 7.0) using a mortar and pestle cooled on ice. The ground tissue was transferred to 15 ml Falcon tubes and centrifuged at 4000 rpm (3095 × *g*) for 10 min at 4 °C. The supernatant was recovered and stored at -80 °C until inoculation.

Virus Inoculation

The virus was rub-inoculated into leaves of *S. rexii* and *N. benthamiana* plants using a pinch of aluminium oxide powder (Sigma) and 10–30 µl viral sap [16]. The entire macrocotyledon (phyllomorph/leaf) was inoculated in *S. rexii*.

Chlorophyll *a* Measurement

Chlorophyll *a* content in leaf tissue was measured 47 days post inoculation, as described previously [19]. For *N. benthamiana*, TRV^{WT}-inoculated green leaves and TRV^{SrPDS}-inoculated white leaves were analysed. For *S. rexii*, green tissue from median lamina and white tissue from proximal lamina of TRV^{SrPDS}-inoculated plants were analysed. Tissue samples were harvested into 1.5 ml Eppendorf tubes, weighed, frozen in liquid nitrogen and ground with metal beads using a TissueLyser II (Qiagen). Aqueous acetone (1 ml of 90% acetone) was added to each tube, vortexed and then centrifuged for 1 min at 13,000 rpm (15,805 × *g*). Absorbance of the supernatants was measured at 630, 645, 663 and 750 nm using a GeneQuant1300 spectrophotometer (GE Healthcare, Chicago, IL, USA) and calculated

as described previously [chlorophyll *a* (μg/ml) = 11.64 e_{663} − 2.16e_{645} + 0.10e_{630}] [17, 19]. Average values for each treatment were calculated from sextuple measurements on biological duplicate samples.

Real-time PCR Analyses

Real-time PCR was carried out on RNA extracted from virus-inoculated *S. rexii* plants 20 days post inoculation (DPI). RNA was extracted as described above and 500 ng used in cDNA synthesis with SuperScript III and Random Hexamer Primers (Thermo Fisher Scientific) following the manufacturer`s protocol. Real-time PCR was carried out on a LightCycler® 480 system (Roche, Basel, Switzerland) with LightCycler® 480 SYBR Green I Master mix (Roche). PCR involved denaturation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 15 s, ending with 95 °C for 5 s and 65 °C for 1 min. The *EF1α* gene of *S. rexii* was used as an internal control and assayed alongside the expression of *SrPDS* and the TRV coat protein (CP) sequence (Table 1). A non-infected plant, and a plant inoculated with wild-type TRV were used as controls for *SrPDS* and TRV expression, respectively. The experiments were carried out in technical triplicates and biological duplicates. The calculation of the relative expression levels and the statistical analyses were carried out with REST [20]. In REST, *p* value from the hypothesis test *P(H1)* represents the probability of the null hypothesis that the difference between the sample and control is due only to chance.

Results

SrPDS VIGS Causes Photobleaching in *Nicotiana benthamiana*

To establish VIGS in *S. rexii*, we chose the *PHYTOENE DESATURASE* (*PDS*) gene as the target for gene silencing. *PDS* encodes an enzyme required for carotenoid biosynthesis, and its silencing results in tissue bleaching through oxidative damage [14, 16]. We identified the *PDS* orthologue in *S. rexii* by BLAST (Fig. S1) and then cloned a 416 bp fragment form the 3′ end of the coding sequence (CDS, Fig. 1a) into the TRV vector to generate pTRV2^{SrPDS} (Fig. 1b). *N. benthamiana* plants infiltrated with a 1:1 mixture of Agrobacterium carrying the TRV1 (pTRV1) and the TRV2 (pTRV2^{WT}) vectors or rub-inoculated with sap containing the wild-type TRV virus (TRV^{WT}) showed typical symptoms of TRV infection: smaller leaves and cell death, but no tissue bleaching (Fig. 1c, e). In contrast, infiltration with an Agrobacterium mixture harbouring pTRV1 and pTRV2^{SrPDS} or rub inoculation with TRV^{SrPDS} viral

sap caused photobleaching (Fig. 1d, f), presumably because the homology between the 3′ region of *SrPDS* and the *PDS* sequence of *N. benthamiana* (82% of 416 nucleotides, 91% of 139 amino acids) was sufficient to trigger *NbPDS* VIGS (Fig. 1a). Chlorophyll *a* content was lower in photobleached TRV^{SrPDS}-inoculated *N. benthamiana* than in green TRV^{WT}-inoculated *N. benthamiana* plants (one-way ANOVA: $p < 0.01$; Fig. 1h). This experiment confirmed that the viral vectors were capable of producing infectious virus and triggering VIGS.

Rub Inoculation of TRV^{SrPDS} Viral Sap Causes Photobleaching in *S. rexii*

Ten weeks after sowing, *S. rexii* plants were subjected to infiltration with Agrobacterium or rub inoculation with viral sap from *N. benthamiana*. Infiltration with buffer, or rubbing with aluminium oxide powder alone resulted in slight decrease in leaf growth (Fig. 2b, c). Similarly, *S. rexii* leaves infiltrated with pTRV1 and $pTRV2^{WT}$ (Fig. 2d) or pTRV1 and $pTRV2^{SrPDS}$ (Fig. 2e) displayed reduced development. However, none of the Agrobacterium-infiltrated leaves showed a photobleaching phenotype (Fig. 2e; Table 2), suggesting that VIGS had not been initiated. No plants died as a result of any of these treatments (Table 2).

In contrast, rub inoculation with plant sap containing virus particles isolated from TRV^{WT} and TRV^{SrPDS}-infected *N. benthamiana* leaves resulted in high plant mortality. 25% of *S. rexii* plants inoculated with TRV^{WT} and 8.3% inoculated with TRV^{SrPDS} died within 26 days post inoculation (DPI) (Table 2; Fig. 2f). Survivors showed more damage or reduced development when compared to other treatments (Table 2; Fig. 2g). These effects were not seen in mock-inoculated plants, suggesting that the plants had been infected with virus and this was responsible for the lethality. Notably, two out of the eleven surviving plants inoculated with TRV^{SrPDS} showed photobleaching by 26 DPI in small patches on the leaf surface, particularly over leaf veins (Fig. 2h, inset). Bleaching became stronger by 42 DPI, but the affected area was displaced to a more distal position by activity of the basal meristem and no more bleached tissue formed proximally (Fig. 2h). No further plants developed photobleaching or died between 26 and 42 DPI. Consistently with *PDS* silencing, the chlorophyll a content was lower in photobleached tissue than in green tissue (one-way ANOVA: $p = 0.01$) in *S. rexii* inoculated TRV^{SrPDS} (Fig. 2i).

Age Dependence of VIGS in *S. rexii*

To test the effect of plant age on the efficiency of VIGS, *S. rexii* plants were inoculated at 5 or 8 WAS. Inoculation with either TRV^{WT} or TRV^{SrPDS} had lethal effects, and significantly more of the plants inoculated at a younger age, 5 WAS, died (Fisher's exact test $p < 0.001$ for independence of survival and age at inoculation with TRV^{SrPDS}; Fig. 3; Table 3). TRV^{SrPDS}-inoculated plants showed tissue bleaching and the frequency was significantly higher in plants that were older at the time of inoculation (60% of inoculated older plants, compared to 16% for younger plants, Fisher's exact test $p = 0.003$; Table 3). Between 8 and 10 WAS, there was no significant difference observed in lethality or proportion of plants showing a photobleaching phenotype (Fisher's exact test $p > 0.05$). Photobleaching became visible after around 2–3 weeks in the proximal part of leaf and was more pronounced over veins. In some cases, it was confined to one half of the leaf (Fig. 3). On the other hand, photobleaching was rarely observed in the median part of the leaf next to the rub inoculated tissue covered with aluminium oxide powder (Fig. 3c, d arrows). As the leaf grew, photobleached tissue was displaced distally by tissue emanating from the activity of the basal meristem, and newly-formed tissue showed less, or no photobleaching (Fig. 3, 31 DPI).

Gene Expression Analyses

Expression of the TRV coat protein gene, *TRV-CP*, was not detected in untreated plants but detected in the virus-inoculated plants 20 DPI (Fig. 3e; Tables S1, S2). The relative abundance of *TRV-CP* in TRV^{SrPDS}-inoculated plants suggested that more viruses existed in the green median region of the lamina compared to the photobleached proximal region showing VIGS ($p < 0.05$; Fig. 3e; Tables S1, S2). This suggests that the virus was able to spread systemically and triggered the RNA interference of the host plant *S. rexii*. As expected, *SrPDS* mRNA was less abundant in the photobleached proximal tissue compared to the green median tissue due to VIGS related RNA interference ($p < 0.05$; Fig. 3f; Tables S1, S2).

Discussion

In this study, TRV-based VIGS was examined in *S. rexii*. Transfer of virus produced in *N. benthamiana* into *S. rexii* by rub inoculation was able to trigger VIGS in over half of plants that were treated. VIGS was not observed in *S. rexii* plants infiltrated directly with Agrobacterium carrying the viral vectors, even though these strains were able to cause VIGS in *N. benthamiana*. The difference might reflect the inability of this Agrobacterium strain to transform *S. rexii* cells efficiently.

VIGS in *S. rexii* appeared to be limited in space and time. Although the presence of viral RNA suggested that TRV was able to infect median leaf tissues next to the rub-inoculated tissue, it did not cause detectable silencing of *SrPDS* in this region. In more basal (younger) tissue, photobleaching caused by VIGS was strongest in cells around the vasculature. This might reflect the fact that long-distance transmission of TRV occurs via the phloem [21], or that the short-interfering RNA (siRNA) responsible for VIGS can also move through phloem from source to sink [22]. In all cases, VIGS was not observed to spread continuously into newly formed leaf tissue basally, although this is expected to be a sink for phloem transport, suggesting that the plants acquired resistance to the virus with time.

The efficacy of VIGS showed a correlation with the age of *S. rexii* seedlings. The younger the plants were infected with TRV the more efficient the silencing of *PDS*. This confirms previous findings in *Arabidopsis* [23]. However, the highest VIGS efficacy was associated with more frequent cell and plant death suggesting that young plants were hypersensitive to TRV infection. Further study is required to understand the underlying molecular mechanism, which may result in more efficient and more stable VIGS in *S. rexii*.

Conclusions

In this study, we examined a VIGS system in the genus *Streptocarpus* to establish a reverse genetic tool for this plant. Two methods were tested, direct viral sap rub application and Agrobacterium infiltration of viral vectors to *S. rexii* leaves. We found that only the former method was able to induce VIGS in *Streptocarpus*, which suggests that this genus is recalcitrant to Agrobacterium-infiltration method successfully used in *N. benthamiana*. In line with our finding, successful Agrobacterium-mediated gene transfer in *Streptocarpus* was reported only through cell suspension culture [24], but not via the standard callus transformation protocol (Y. Hoshino pers. comm.) Thus, the positive VIGS result for *Streptocarpus* shown here is of high significance. We further showed that *SrPDS* is an effective reporter gene for VIGS in S. rexii and could be used to develop a multiplexed VIGS system or alternative VIGS vectors.

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Author Contributions All authors contributed to the study conception and design, and manuscript preparation. Data collection and analyses were performed by Yue Fei and Kanae Nishii, with supervision of Attila Molnar, Andrew Hudson, and Michael Möller. All authors read and approved the final manuscript.

Data Availability The *SrPDS* sequence generated and/or analysed during the current study are available in the BLAST repository [NCBI GenBank ID MT127415; https://www.ncbi.nlm.nih.gov].

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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388 **Table 1** Primer sequences used in this study

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Gene	Direction	Sequence (5' - 3')	Usage
SrPDS	Forward	TGG TTT GAC AGG AAG CTG AAG A	Vector construction
SrPDS	Reverse	GAC AGG ACA GCA CCT TCC AT	Vector construction
SrPDS	Forward	ACG AGG GGG ACT GGT ATG AA	Realtime PCR
SrPDS	Reverse	AAT GGT GCG GGC AAA ACT TC	Realtime PCR
SrEF1α	Forward	CAC CTT TGC CCC TAC TGG TT	Realtime PCR
SrEF1α	Reverse	AGC CTC GCT TGA GAT CCT TG	Realtime PCR
TRV-CP	Forward	TGG GTT ACT AGC GGC ACT GA	Realtime PCR
TRV-CP	Reverse	GCT CGT CTC TTG AAC GCT GA	Realtime PCR
TRV-CP	Forward	GTT CAG GCG GTT CTT GTG TGT C	Sequencing
TRV-CP	Reverse	TTA CCG ATC AAT CAA GAT CAG	Sequencing

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391 **Table 2** Lethality and phenotype observations of different VIGS treatments 26 days post inoculation in
392 *Streptocarpus rexii*

393

Treatment	Alive	Dead	Photobleaching phenotype	No. of plants treated
No treatment	4 (100.0%)	0 (0.0%)	0 (0.0%)	4
Buffer infiltration	4 (100.0%)	0 (0.0%)	0 (0.0%)	4
Aluminium oxide powder rubbing	4 (100.0%)	0 (0.0%)	0 (0.0%)	4
pTRV1 & pTRV2^{WT} Agrobacterium infiltration	4 (100.0%)	0 (0.0%)	0 (0.0%)	4
pTRV1 & pTRV2^{SrPDS} Agrobacterium infiltration	12 (100.0%)	0 (0.0%)	0 (0.0%)	12
TRV^{WT} viral sap rub inoculation	6 (75.0%)	2 (25.0%)	0 (0.0%)	8
TRV^{SrPDS} viral sap rub inoculation	11 (91.6%)	1 (8.3%)	2 (16.7%)	12

394 Plants were inoculated 10 weeks after sowing

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397 **Table 3** Lethality and phenotype observations of TRV^{WT} or TRV^{SrPDS} sap rub inoculation 20 days post
398 inoculation in *Streptocarpus rexii*

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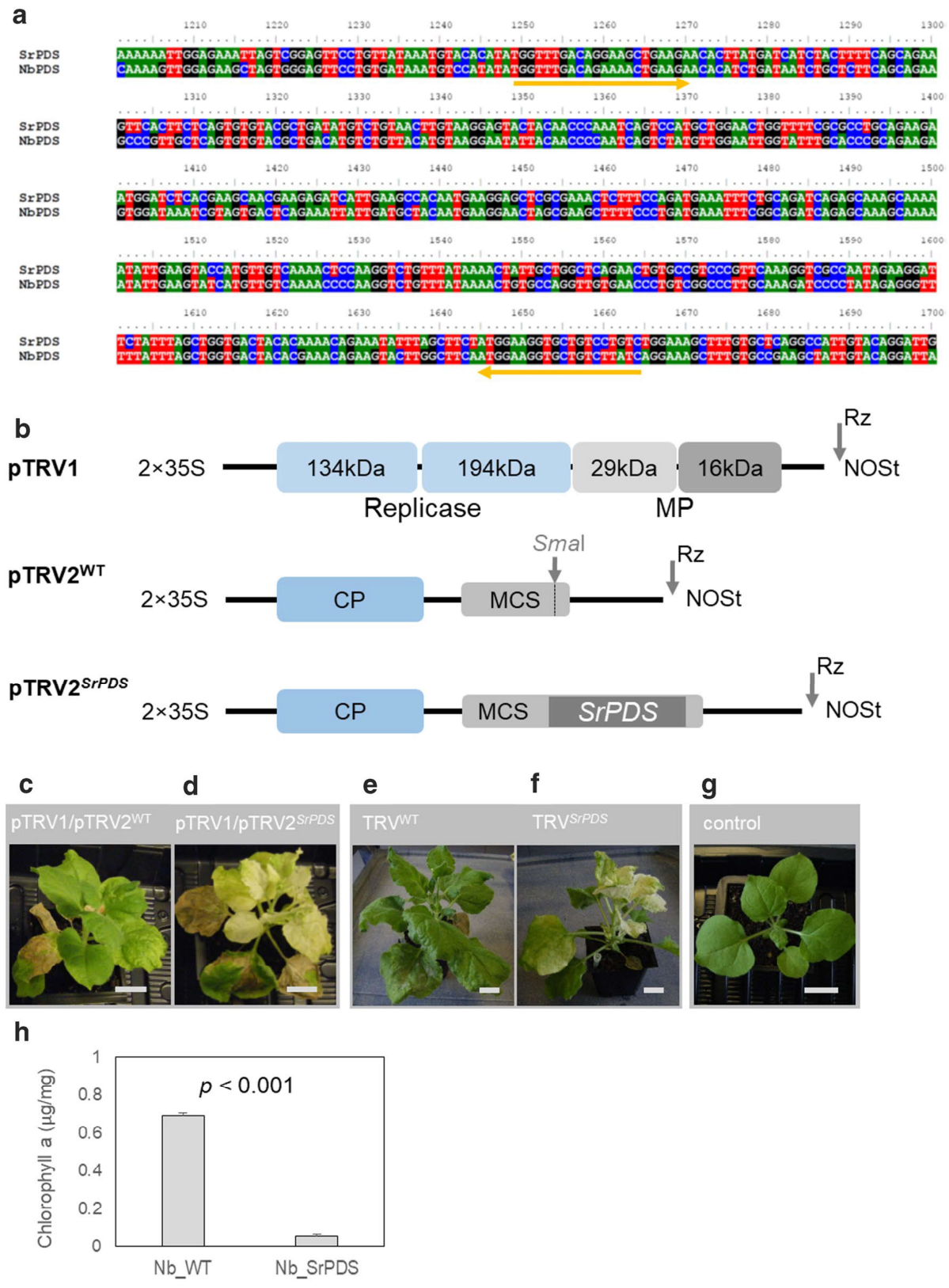
Treatment	WAS	Alive	Dead	Photobleaching phenotype	No. of plants treated
No treatment	5	3 (100.0%)	0 (0.0%)	0 (0.0%)	3
No treatment	8	3 (100.0%)	0 (0.0%)	0 (0.0%)	3
TRV^{WT} viral sap rub inoculation	5	8 (88.9%)	1 (11.1%)	0 (0.0%)	9
TRV^{WT} viral sap rub inoculation	8	7 (77.8%)	2 (22.2%)	0 (0.0%)	9
TRV^{SrPDS} viral sap rub inoculation	5	13 (54.2%)	11 (45.8%)	4 (16.6%)	24
TRV^{SrPDS} viral sap rub inoculation	8	46 (95.8%)	2 (4.2%)	29 (60.4%)	48

400 Plants were inoculated 5 or 8 weeks after sowing (WAS)

401

Fig. 1 A pTRV2^{SrPDS} construct induces photobleaching in *Nicotiana benthamiana*. **a** Partial sequence of *Phytoene desaturase* (*PDS*) nucleotide gene sequences isolated from *Streptocarpus rexii* (*SrPDS*) aligned with *Nicotiana benthamiana PDS* (*NbPDS*: EU165355). Arrows indicate primer positions used for the preparation of the pTRV2^{SrPDS} construct. b Schematic illustrations of the vector constructs used in this study. *SrPDS* fragment was inserted in pTRV2 vector. MP: movement protein, CP: coat protein, MCS: multiple cloning site, Rz: self-cleaving ribozyme, NOSt: nopaline synthase terminator. **c–g** Phenotypes of TRV VIGS in *Nicotiana benthamiana*. Agrobacterium infiltrated with **c** pTRV1/pTRV2^{WT}, and d pTRV1/pTRV2^{SrPDS} 27 days after infiltration; **e** TRV^{WT} viral sap and **f** TRV^{SrPDS} rub-inoculated *N. benthamiana* 22 days post inoculation. **g** Control plant without treatment. Scale bars = 2 cm. **h** Chlorophyll *a* measurements 47 days post inoculation. Nb_WT: TRV^{WT} inoculated N. benthamiana, Nb_SrPDS: TRV^{SrPDS} inoculated *N. benthamiana*. *p* values are from a one-way ANOVA


a
SrPDS
NbPDS
b
pTRV1
2×35S
134kDa
194kDa
29kDa
16kDa
Rz
NOSt
Replicase
MP
pTRV2WT
SmaI
CP
MCS
pTRV2SrPDS
SrPDS
c
d
e
f
g
pTRV1/pTRV2WT
pTRV1/pTRV2SrPDS
TRVWT
TRVSrPDS
control
h
Chlorophyll a (µg/mg)
p < 0.001
Nb_WT
Nb_SrPDS

417

418

419

Fig. 2 Phenotypes of different VIGS treatments in *Streptocarpus rexii*. Plants treated ten weeks after sowing and photographed 42 days post inoculation (DPI). **a** Control, no treatment. **b** Agrobacterium transformation buffer infiltration. **c** Aluminium oxide powder rubbing. **d** pTRV1&pTRV2^{WT} Agrobacterium infiltration. **e** pTRV1&pTRV2^{SrPDS} Agrobacterium infiltration. **f, g** TRV^{WT} viral sap rub inoculation. This treatment resulted in lethal damage (**f**) or suppression of lamina growth (**g**). **h** TRV^{SrPDS} viral sap rub inoculation. Inset is the image of the same plant earlier in development at 26 DPI. Arrows indicate photobleached patches on leaves. See also Table 2. Scale bars = 2 cm. i Chlorophyll *a* measurement 47 days post inoculation. Sr_SrPDS_G: green tissue of TRV^{SrPDS} inoculated *S. rexii*, Sr_SrPDS_W: white (photobleached) tissue of TRV^{SrPDS} inoculated *S. rexii*. *p* values are from a one-way ANOVA

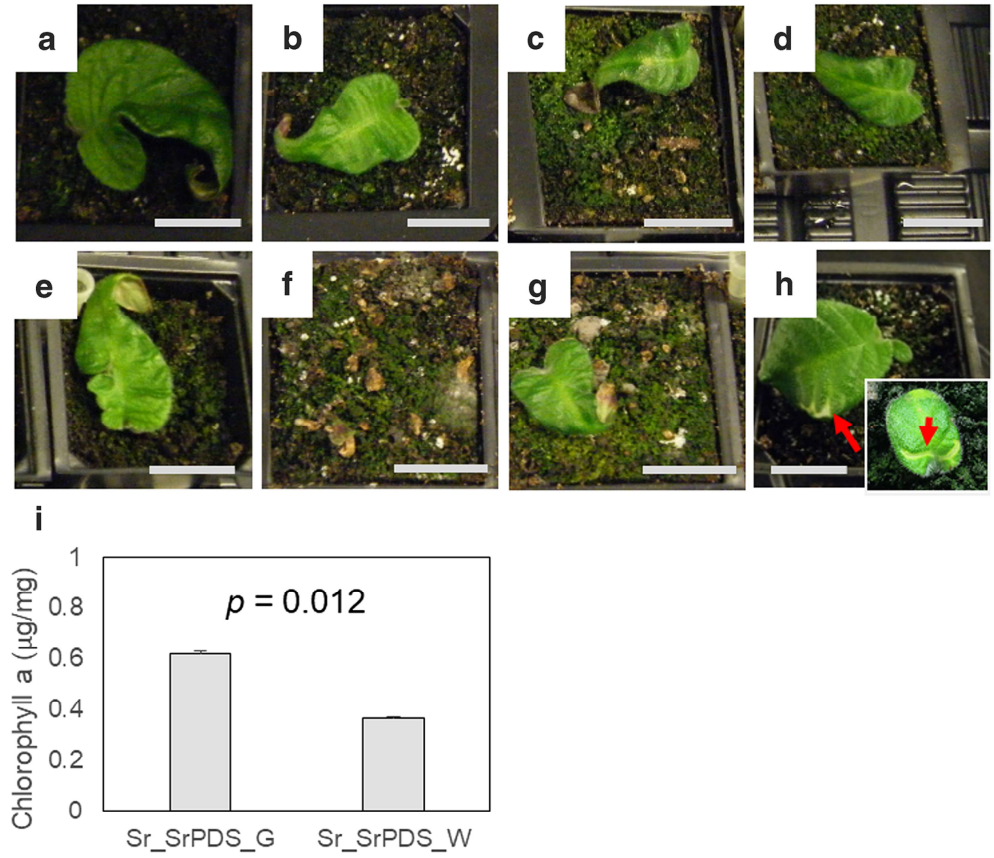
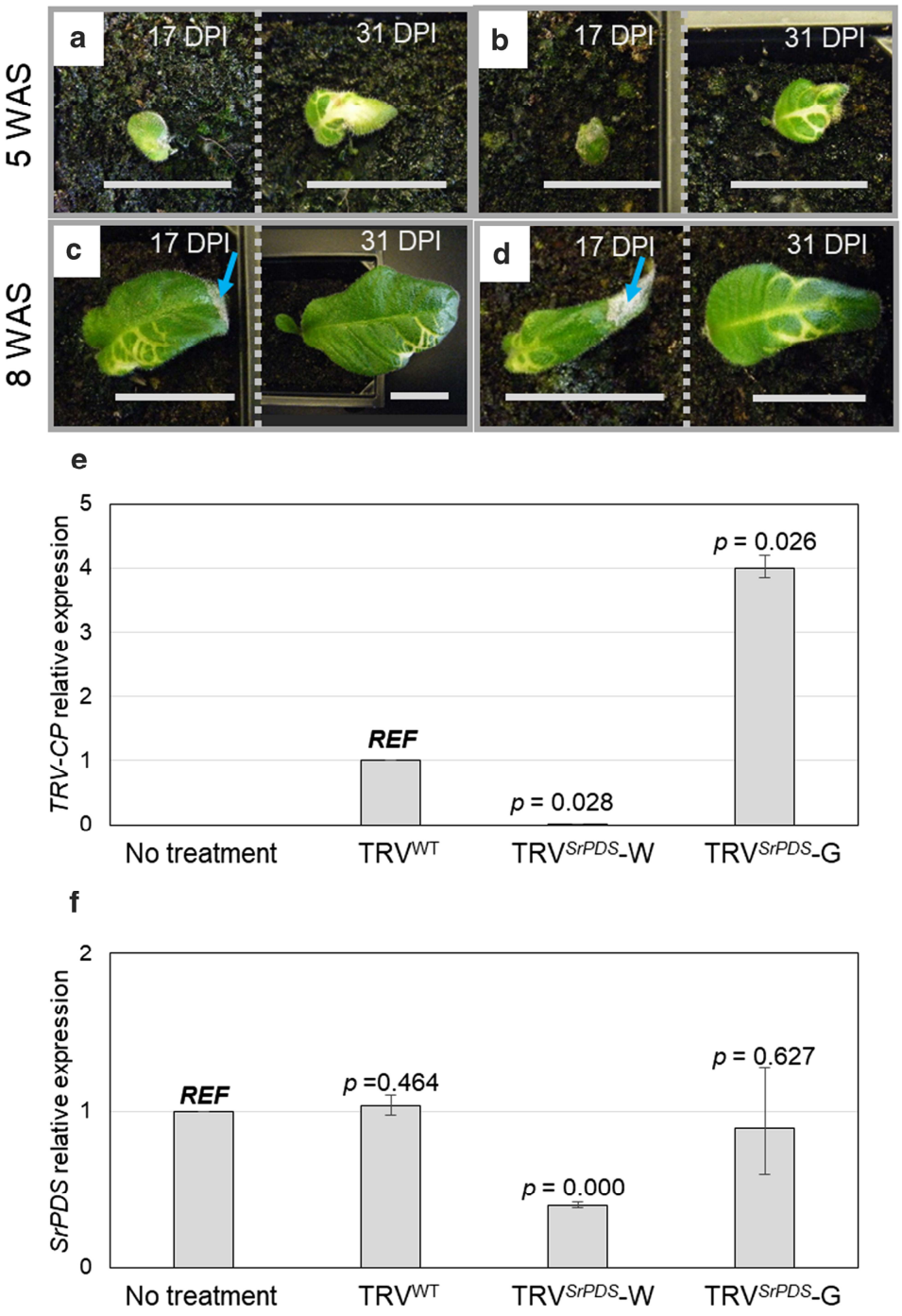


Fig. 3 Age effects of VIGS in *Streptocarpus rexii*. Phenotypes of *S. rexii* plants inoculated with TRV^{SrPDS} plant sap rubbing at 5 and 8 weeks after sowing (WAS). Images taken 17 and 31 days post inoculation (DPI). **a**, **b** Virus inoculation at 5 WAS. **a** Plant exhibiting photobleaching in the distal part. **b** Plant showing photobleaching in the veins. **c**, **d** Virus inoculation at 8 WAS. **c** Plant with photobleaching in the veins, but only on one side of the lamina. **d** Plant showing the photobleaching phenotype in veins in the distal region of the lamina. Neighbouring images represent 17 DPI (left) and 31 DPI (right) of the same plant. Arrows indicate residues of aluminium oxide powder on the lamina surface after rub inoculation, indicating the lamina size at inoculation and the location of the rub inoculation. See also Table 3. Scale bars = 2 cm. **e**, **f** Relative gene expression of *TRV-CP* (**e**) and *SrPDS* (**f**) in *Streptocarpus rexii* plants, 20 DPI, inoculated with viral sap 8 weeks after sowing. The relative expression level was calculated with TRV^{WT}-inoculated plants as reference for *TRV-CP* and untreated plant as reference for *SrPDS*. *REF*: the reference samples (Expression = 1). *EF1α* gene expression used as internal control. The *p* values from hypothesis tests *P(H1)*, represent the probability of the null hypothesis that the difference between the sample and control is due only to chance (see Tables S1, S2). TRV^{WT}: whole leaf, sap rub infected with TRV^{WT}, TRV^{SrPDS}-W: photobleached tissue of proximal part of the leaf, sap rub infected with TRV^{SrPDS}, TRV^{SrPDS}-G: green tissue of distal part of the leaf, sap rub infected with TRV^{SrPDS}. Graphs drawn from the 1st replicate of data shown in Tables S1 and S2


a
17 DPI
31 DPI
b
17 DPI
31 DPI
5 WAS
c
17 DPI
31 DPI
d
17 DPI
31 DPI
8 WAS
e
TRV-CP relative expression
5
4
3
2
1
0
REF
p = 0.028
p = 0.026
No treatment
TRVWT
TRVSrPDS-W
TRVSrPDS-G
f
SrPDS relative expression
2
1
0
REF
p =0.464
p = 0.000
p = 0.627
No treatment
TRVWT
TRVSrPDS-W
TRVSrPDS-G

455

456

457

Fig. S1

Phytoene desaturase (*PDS*) nucleotide gene sequences isolated from *Streptocarpus rexii* (*SrPDS*) aligned with *Nicotiana benthamiana PDS* (*NbPDS*: EU165355). Arrows indicate primer positions used for the preparation of the pTRV2^{SrPDS} construct

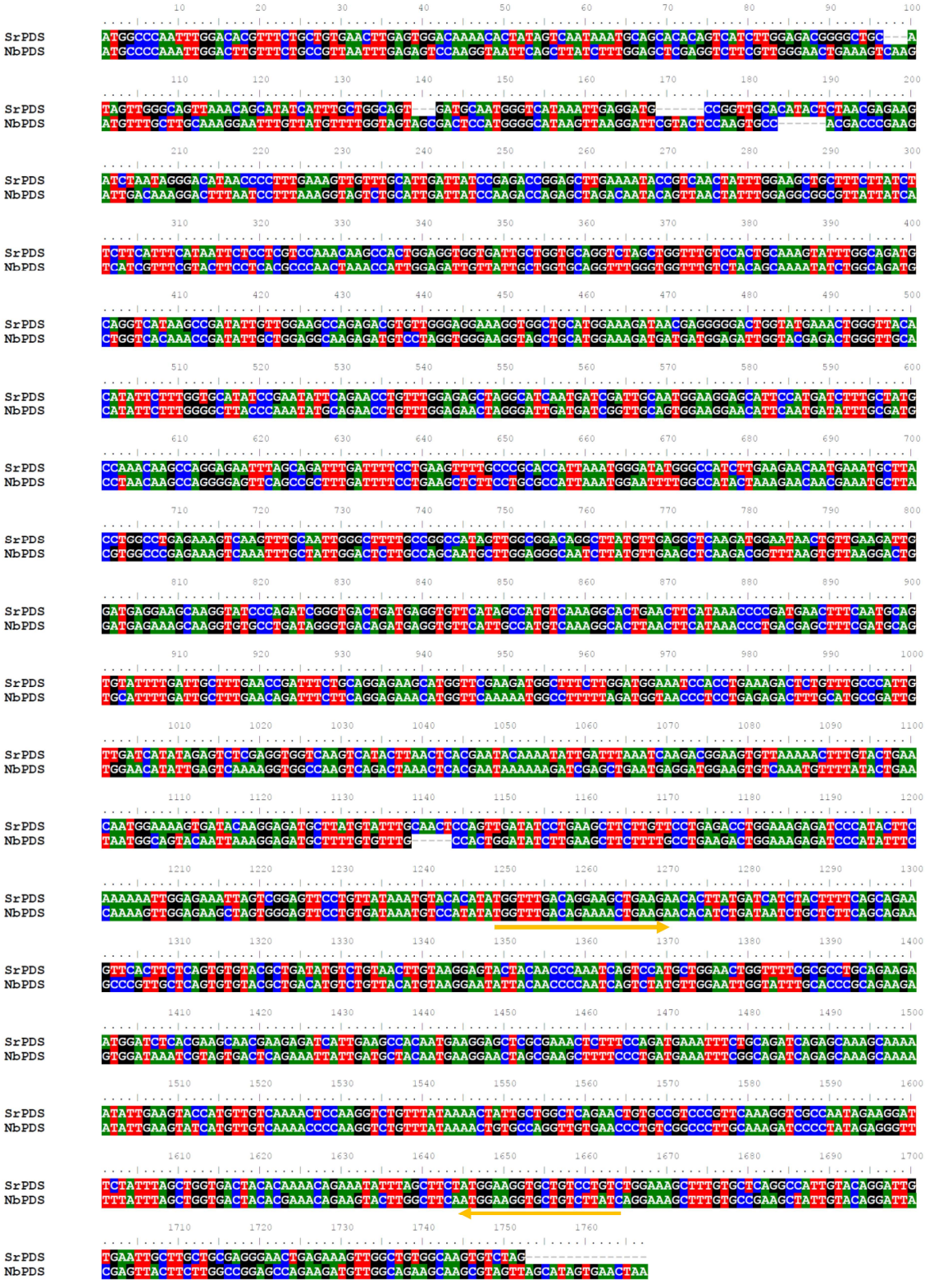


Table S1 Relative gene expression of *TRV-CP* and *SrPDS* in *Streptocarpus rexii* plants, 20 DPI, inoculated with viral sap 8 weeks after sowing. The relative expression level was calculated with TRV^{WT} inoculated plants as reference for *TRV-CP* and untreated plant as reference for *SrPDS*. Asterisks indicate the reference samples (Expression = 1.000). *EF1α* gene expression used as internal control. The hypothesis test *P(H1)* in REST, represents the probability of the null hypothesis that the difference between the sample and control is due only to chance.

			TRV-CP relative expression				*SrPDS* relative expression			
Sample	Area	Tissue colour	Expression	Std. error	95% C.I.	*P*(*H1*)	Expression	Std. error	95% C.I.	*P*(*H1*)
No treatment	Whole	Green	Not detected	-	-	-	1.000*	-	-	-
TRV^{WT}	Whole	Green	1.000*	-	-	-	1.030	0.975 - 1.099	0.929 - 1.110	0.464
TRV^{SrPDS} 1	Proximal	Photobleached	0.009	0.008 - 0.009	0.008 - 0.010	0.028	0.402	0.386 - 0.423	0.370 - 0.433	0.000
	Median	Green	3.991	3.852 - 4.197	3.692 - 4.252	0.026	0.891	0.600 - 1.276	0.572 - 1.329	0.627
TRV^{SrPDS} 2	Proximal	Photobleached	0.002	0.002 - 0.002	0.002 - 0.002	0.031	0.849	0.690 - 0.971	0.657 - 0.995	0.000
	Median	Green	0.016	0.015 - 0.017	0.015 - 0.017	0.037	2.645	2.430 - 2.951	2.372 - 3.074	0.056

Table S2 Relative gene expressions of proximal photobleached tissue using median green tissue as reference (Expression = 1.000), in TRV^{SrPDS} inoculated *Streptocarpus rexii* plants 20 DPI, inoculated with viral sap 8 weeks after sowing. *EF1α* gene was used as internal control. Each sample was calculated separately. The hypothesis test *P(H1)* in REST represents the probability of the null hypothesis that the difference between the gene expression level of proximal photobleached tissue and median green tissue is due only to chance.

Sample	Gene	Relative expression	Std. error	95% C.I.	Result	*P*(*H1*)
TRV^{SrPDS} 1	*TRV-CP*	0.002	0.002 - 0.002	0.002 - 0.002	Down	0.019
	SrPDS	0.452	0.319 - 0.676	0.301 - 0.703	Down	0.048
TRV^{SrPDS} 2	*TRV-CP*	0.106	0.091 - 0.128	0.080 - 0.136	Down	0.048
	SrPDS	0.321	0.271 - 0.381	0.237 - 0.388	Down	0.000